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OCCURRENCE OF CALCIUM OXALATE AND OXALATE-UTILIZING BACTERIA IN *ECHINODONTIUM TINCTORIUM* DECAY ZONES IN *ABIES CONCOLOR*[1] [2]

by

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ABSTRACT

Concentrations of Ca and oxalate were measured separately in both apparently sound and decayed *Abies concolor* wood. *Echinodontium tinctorium*, the fungus causing the decay, is capable of producing oxalate *in vitro*. Although not statistically significant, highest concentrations of both Ca and oxalate occurred in incipient and advanced decay zones. The average ratio of Ca to oxalate in these zones was 1:1.85, close to the ratio of 1:2.2 in pure Ca oxalate. Bacteria capable of utilizing Ca oxalate were isolated from decay zones of two trees infected with *E. tinctorium* and from the uninfected control tree. It is postulated that recycling of Ca and other cations by degradation of oxalates by bacteria may play an important role in decay of wood by fungi, however, additional studies are needed to demonstrate this hypothesis. An additional hypothesis we propose is that production of oxalic acid may enhance the rate of pectin decomposition by *E. tinctorium*.

KEYWORDS: Heart rot, white fir, decay process, discoloration, Indian paint fungus, deterioration.

INTRODUCTION

The Indian paint fungus, *Echinodontium tinctorium* Ell. and Ev. is responsible for the greatest decay losses in true firs and hemlocks in western North America (Boyce 1961). Recent studies indicate an interrelated community of microorganisms is associated with hymenomycetous fungi that cause decay (Shigo 1967). Many microorganisms, including bacteria, have been consistently isolated from decay caused by *E. tinctorium* (Aho 1976, Hudson 1972). The roles of bacteria in the decay process, though relatively unknown, may be mutualistic (Cosenza et al. 1970). For example, Aho et al. (1972) isolated bacteria capable of fixing atmospheric nitrogen from decay associated with *E. tinctorium* and other fungi in white fir (*Abies concolor* (Gord. and Glen.) Lindl.). Bacterial fixation of nitrogen may be a source of nitrogen needed by fungi to decay wood, a substrate of high C/N ratio (Merrill and Cowling 1966).

Fungi, in a variety of natural habitats, produce oxalic and other organic acids as a part of carbohydrate metabolism (Foster 1949). Production of oxalic acid by *Sclerotium rolfsii* (Sacc.) facilitates cell wall decomposition by precipitating calcium from cell wall pectates and facilitating polygalacturonase activity (Bateman and Beer 1965). Bacteria capable of utilizing relatively insoluble oxalates, such as calcium oxalate, have been isolated from earthworms, various types of soils, and dungs (Chandra and Shethna 1975). Such bacteria, however, have not been isolated from decomposing wood.

The objectives of this exploratory study were to determine the concentrations of oxalate and calcium in sapwood and heartwood and various decay zones caused by *E. tinctorium* in white fir, to isolate bacteria capable of utilizing calcium oxalate as an energy source from the wood and decay zones, and to determine whether or not *E. tinctorium* produces oxalic acid in pure culture. An additional objective was to determine whether or not oxalate-utilizing bacteria are present in decay caused by other fungi in western hemlock and Sitka spruce in a coastal Oregon forest.

METHODS AND MATERIALS

Field Collections

Wood samples were collected from two white firs bearing *E. tinctorium* sporophores and from one tree without decay located near Porcupine Ridge (elevation 1 200 m) in the Butte Falls Ranger District, Rogue River National Forest. Two samples, 60 cm in length, were cut from the bole of each tree. The samples were taken from the trees at heights of approximately 4.5 m and 10.5 m above ground. The wood sections were placed in individual plastic bags and taken to Corvallis on the same day. They were stored approximately 60 h at 2°C before being used in the experiments.

Advantage was taken of an unrelated study made at the Cascade Head Experimental Forest, near Otis, Oregon to test for presence of calcium oxalate decomposing bacteria in wood decayed by fungi other than *E. tinctorium*. Discolored and decayed wood blocks (7.5 cm^3) were removed from 53 western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and 99 Sitka spruce (*Picea sitchensis* (Bong.) Carr.) boles bearing 15-year-old injuries, 12.5 cm wide by 50 cm long. Wounds had not been treated with chemicals, and one wound was sampled per tree.

Sample blocks were obtained from the wood behind the face of the bottom, middle, and top of each wound. They were placed in individual plastic bags and taken to a field laboratory at Cascade Head Experimental Forest where isolations were made. Two isolations were taken from each block.

Laboratory Tests

The bark was removed from the 60-cm white fir sections, and a 7.5-cm-long disk was cut from the center of each. We attempted to isolate bacteria and decay fungi and to determine calcium and oxalate concentrations in sapwood, early, incipient, and advanced decayed wood of the two infected trees and in sound sapwood and heartwood zones of the uninfected control tree.

Echinodontium tinctorium isolates from decayed wood in white fir were tested for their ability to produce oxalate in pure culture on an artifical medium. Mycelia of *E. tinctorium* were put on a nylon mesh (Nitex 276) applied to the malt agar surface and incubated at 18°C for 2 weeks. Mycelial mats were collected from the nylon mesh, lyophilized, and introduced into 100- X 16-mm I.D. glass tubes with teflon-lined screw caps. Three ml of 5 percent HCI-methanol solution were added to each tube. Oxalate concentration was measured using the gas chromatographic method of Mee and Stanley (1973).

Isolation of bacteria and fungi.--Two methods were used to isolate calcium oxalate-utilizing bacteria. First, wood plugs (7.5 cm long by 1.2 cm diam) were removed aseptically from each wood and decay zone with a tubular chisel, placed in sterile rubber tubing, and subjected to 200 to 350 Kg/cm^2 of pressure in a hydraulic press (Aho et al. 1974, Knutson 1970). One ml of expressed sap was immediately diluted in 9 ml of sterile distilled water and serially diluted to 1/1000. One-tenth ml of sap and of each dilution (1/10, 1/100, 1/1000) from each wood and decay zone was pipetted into each of three plates containing a calcium oxalate medium modified slightly from that reported by Jayasuriya (1955). Yeast extract concentration was reduced from 0.1 percent to 0.01 percent (v/v) and 3 percent agar (Difco) was substituted for Davis New Zealand agar. The inoculum was spread over the surface of the medium with a sterile glass rod. The plates were incubated at 25°C and checked periodically for growth. A clear zone developed around bacteria capable of utilizing calcium oxalate (Jayasuriya 1955).

Second, when it was not possible to squeeze sap from various white fir wood and decay zones, small wood chips (about 3 mm^3) were removed aseptically from freshly exposed surfaces (Aho et al. 1974), placed on the calcium oxalate medium in petri plates, and incubated at 25°C. The same technique was used to isolate decay fungi except that the small wood chips were placed on 2.5 percent malt agar (Difco) in culture tubes and incubated at 20°C.

The discolored and decayed wood samples behind injuries in western hemlock and Sitka spruce trees were treated similarly. Each was carefully split to expose fresh surfaces. Six wood chips (two each from the blocks taken from the bottom, middle, and top of the scar) were plated on the calcium oxalate medium and incubated at 25°C. Two chips from each sample block were also placed on malt agar slants.

Concentration of oxalic acid.--Pieces of wood from each decay zone, weighing at least 10 g, were carefully removed from the white fir disks, lyophilized, and ground in a Wiley mill to pass a 40-mesh screen. Concentration of oxalate present in each sample was determined by the gas chromatographic method of Mee and Stanley (1973). Calcium oxalate is solubilized with IN HCI which also catalyzes a reaction between methanol and oxalate forming dimethyl oxalate. Concentration of oxalate is determined by comparison with standard concentrations of dimethyl oxalate.

Concentration of calcium.--The remaining portions of each white fir disk were carefully separated by stage of decay, oven-dried to constant weight at 65°C, and ground in a Wiley mill to pass a 40-mesh screen. Samples were ashed at 500°C, ash was taken up in HCI, and calcium concentration determined by spark emission spectroscopy (Chapman and Dickson 1974).

Statistical Analyses

Mean and standard error of the mean were calculated for calcium and oxalate concentrations for sound and decayed wood in each of the three trees by pooling the observations for samples at two heights.

RESULTS AND DISCUSSION

Isolation of Bacteria and Fungi

Bacteria capable of utilizing calcium oxalate as an energy source were isolated from all three white fir study trees. None were found in the sapwood or advanced decay zones but were isolated from: (1) early and incipient decay in both sections from tree 1, (2) incipient decay at 10.5 m in tree 2, and (3) the inner heartwood zone of tree 3 at 10.5 m. The latter heartwood zone was not visibly decayed, but was slightly discolored. No fungi could be isolated from it. The only decay fungus isolated from decayed wood in trees 1 and 2 was *E. tinctorium.* Bacteria and black non-hymenomycetous fungi were also isolated from decay zones in these trees.

Due to the low numbers of calcium oxalate-utilizing or other bacteria found in this study, it was not possible to make an accurate population estimate. We attribute this result to time of year (winter), low moisture content of the wood, and most importantly, to the high concentrations of phenolic compounds present in sap expressed from *E. tinctorium* decay (Aho et al. 1974).

Conclusive evidence that these bacteria are commonly associated with discolored and decayed wood in living trees was obtained by isolations from wounded western hemlock and Sitka spruce. Calcium oxalate-utilizing bacteria were isolated from at least one of six chips from 62 percent of the wood samples behind scars on 53 hemlock and nearly 60 percent of the 99 wounds on spruce. These data indicate that calcium oxalate-decomposing bacteria are present in decayed wood other than that caused by *E. tinctorium.* To the best of our knowledge this is the first report of the presence of calcium oxalate-utilizing bacteria in living trees. No attempt was made to identify these bacteria from white fir, western hemlock, or Sitka spruce, but all were gram-negative rods.

Concentrations of Calcium and Oxalate

Oxalate was found in all *E. tinctorium* isolates tested. Concentrations in two cultures were 0.8 and 0.5 percent of freeze-dried weight. This confirms, at least *in vitro,* that the white rot fungus *E. tinctorium* has a physiological capability to produce oxalate and in concentrations similar to that found in rhizomorphs produced by *Fomitopsis pinicola* Swarz. & Franz in decayed Douglas-fir heartwood (Cromack et al. 1977). Other researchers have shown that both white and brown rot fungi produce relatively large amounts of oxalate (Henningsson 1965, Milova 1973, Nord and Vitucci 1947).

Oxalate was detected in all wood and decay zones tested (table 1). Concentrations of oxalate were higher, though not statistically significant, in the incipient and advanced decay zones of the two infected trees than in sound sapwood or heartwood zones of the uninfected control tree. Calcium concentrations corresponded to those of oxalate, also being nonsignificantly higher in the incipient and advanced decay zones (table 1). The average ratio of Ca to oxalate in incipient and advanced decay stages was 1:1.85 (0.25 S.E., n = 7 samples); whereas, in pure Ca oxalate it is 1:2.2. The average calcium concentrations in the incipient and advanced decayed wood are in excess of those needed to form a precipitate with all of the oxalate anion present. Due to the limited sample size, it is quite possible that in some locations within individual trees, adequate Ca is not present to precipitate all the

Table 1--Concentrations of oxalate and calcium in sound wood and *Echinodontium tinctorium* decay zones in white fir

Tree number	Wood or decay zone	Oxalate[1]		Calcium[1]	
		Concentration (% dry wt.)	Standard error	Concentration (% dry wt.)	Standard error
1	Sapwood	0.146	0.011	0.09	0.010
	Early decay	.124	.011	.10	.005
	Incipient decay	.807	.020	.46	.005
	Advanced decay	.382	.180	.32	.150
2	Sapwood	.421	.010	.13	.030
	Early decay	.471	.050	.18	.010
	Incipient decay	.571	.025	.27	.070
	Advanced decay	.521[2]	--	.23[2]	--
3	Sapwood	.068	.022	.08	.005
	Outer heartwood	.113	.022	.26	.040
	Inner heartwood	.068	.022	.17	.090

[1] Average of two measurements made at different heights (4.5- and 10.5-m) in each tree.

[2] Advanced decay stage present only in the 4.5-m sample in tree 2.

oxalate anion present. Other studies have shown that calcium is concentrated in decayed wood by fungi (Johansson and Theander 1974, Rennerfelt and Tamm 1962, Safford et al. 1974, Tatter et al. 1971), including *E. tinctorium* (Ellis 1956) suggesting that oxalate can be accumulated by wood decay fungi.

Concentration trends of calcium and oxalate in sapwood and inner and outer heartwood (Tree 3) were similar. Highest concentrations of calcium occurred in wood containing the highest levels of oxalate (table 1). The presence of oxalate in sound woody tissue should not be considered unusual since it is known that trees also naturally produce this compound (Esau 1967). Bacteria have been isolated from apparently healthy woody tissues (Aho and Hutchins 1977), including sapwood (Knutson 1973, Shortle and Cowling 1978). Conceivably, oxalate produced by a tree could be a substrate for some of these bacteria.

We suggest that the high concentrations of calcium and oxalate in incipient and advanced decayed wood, and the presence of bacteria capable of utilizing calcium oxalate as a source of energy aid microorganisms attacking cellulose and lignin components of wood by recycling cations. Decay fungi, such as *E. tinctorium*, or associated non-hymenomycetous fungi, produce oxalic acid in the degradation of wood. Calcium and oxalate precipitate to form a sparingly soluble compound; other cations form complexes with oxalate anions. Neutralization of free oxalic acid by formation of calcium oxalate or by formation of complexes with other cations such as Fe or Al would maintain a higher pH in decayed wood than would free oxalic acid thus favoring fungal growth. Decomposition of Ca oxalate by bacteria would tend to increase microsite pH, with formation of soluble Ca $(HCO_3)_2^-$. Jayasuriya (1955) found pH to increase from 7 to 9.5 with bacterial decomposition of K oxalate. The Ca $(HCO_3)_2^-$ can react with cation exchange sites or with the oxalate anion to form additional Ca oxalate, thus completing a cycling of Ca. Calcium cycling within the three decay zones may be analogous to recycling of Ca within soil profiles, where both fungal production of the oxalate anion and bacterial decomposition of oxalates may considerably influence the weathering rates of cations, causing release of P from Fe and Al hydroxy phosphates (Graustein et al. 1977).

In the process of wood decay, oxalic acid production by *E. tinctorium* may enhance pectinase activity by the same chemical mechanism suggested by Bateman and Beer (1965); *E. tinctorium* is known to produce oxalic acid (this study) and pectinase *in vitro* (Mayers 1932).

We hope the results of this study will stimulate additional research to define and clearly demonstrate the roles of fungi and bacteria in accumulation and utilization of oxalate and calcium (and other cations), respectively, in the decay process.

Answers to the following questions will help in our understanding of the process of wood decay in living trees:

1. What is the role of Ca oxalate utilizing bacteria in the wood decay process by *E. tinctorium* and other decay fungi, including effects on pH and cation exchange processes?
2. What role does oxalic acid production by *E. tinctorium* and other decay fungi have in regulating rate of pectin decomposition?
3. Can nitrogen-fixing bacteria, commonly found in decaying wood, also utilize Ca oxalate?
4. Is atmospheric nitrogen fixed by bacteria utilized by fungi decayed wood? Is rate of decay increased by presence of nitrogen-fixing and Ca oxalate utilizing bacteria?

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